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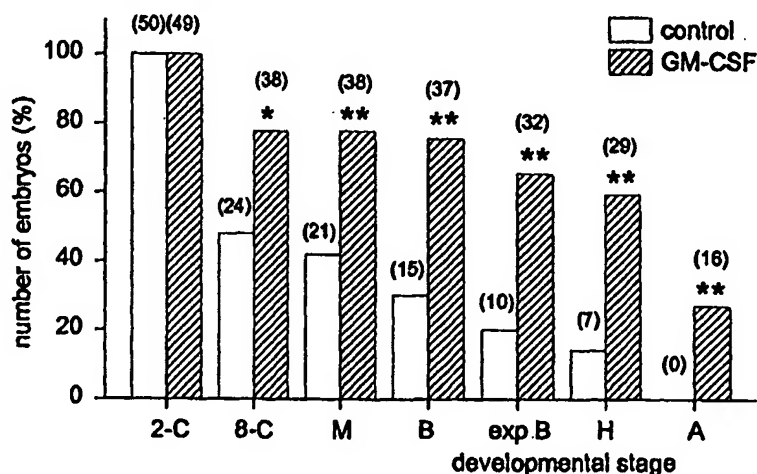
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(54) Title: METHOD AND MEDIUM FOR IN VITRO CULTURE OF HUMAN EMBRYOS

(57) Abstract

Disclosed is a medium for the propagation of early stage embryos to blastocyst stage. The medium contains an effective amount of human GM-CSF to increase the percentage of pre-blastocyst embryos which develop to transfer ready blastocysts. Also disclosed is a method of growing early stage human embryos to transfer ready blastocysts. The method includes the step of incubating the embryos *in vitro* in a culture medium containing an effective amount of human GM-CSF for a time and under conditions to increase the proportion of transfer ready blastocysts. An IVF program that includes the method of growing early stage human embryos to transfer ready blastocysts is also disclosed.



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METHOD AND MEDIUM FOR IN VITRO CULTURE OF HUMAN EMBRYOS

Infertility is a great concern to many couples who wish to conceive. The proportion of couples that are unable to conceive naturally is remarkably high. In the USA it is said that
5 some 10-15% of couples of reproductive age are unable to have children, whereas in the United Kingdom the proportion has been estimated at 14%.

In the last 20 years or so some hope has been held out to infertile couples with the development of *in vitro* fertilisation (IVF) techniques. These IVF techniques generally
10 take the form of stimulating the female to ovulate, contacting collected ova with sperm *in vitro* and introducing fertilised ova into the uterus. Multiple variations of this general process also exist. Despite considerable research and technical advances in the IVF field the rate of successful pregnancy following IVF treatment is still quite low and is in the order of 15 to 25% per cycle.

15 Undertaking an IVF program often causes great anguish, especially where there is no resultant successful pregnancy. It is presently believed that the poor success rate for IVF treatment is due to an extraordinarily high rate of early embryonic loss or implantation failure (Weinberg *et al.*, 1988; Lenton *et al.*, 1988).

20 The low efficacy of IVF, together with its high cost and the associated psychological trauma from repeated treatment failures make it desirable that improvements are made to the procedure. Current methods of increasing pregnancy rates during IVF treatment include placing multiple embryos (2-5) into the uterine cavity. This is not always
25 successful, and also carries with it a higher risk of multiple pregnancy.

In most *in vitro* fertilisation units embryos are transferred to the uterus 2 days after fertilisation (4-8 cells). One view is that the use of embryos at this early stage may contribute significantly to the low pregnancy outcome of IVF programs, and that it is more
30 desirable to use embryos at the blastocyst stage reached at day 5 – 7 of culture. The advantages suggested include improved synchronisation between embryo and uterus and the ability to select better quality embryos over the longer culture period. Blastocyst transfer may also help reduce the number of multiple births resulting from IVF, through allowing the selection of fewer numbers of highly competent embryos per transfer.

35 Unfortunately in standard culture media the majority of embryos (about 75%) fail to develop beyond the 4-8 stage. Nevertheless with certain clinical indications implantation of human embryos is performed at the blastocyst stage despite the low proportions of

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embryos that develop to blastocyst. Some recent studies have used co-culture techniques whereby embryos are co-cultured with feeder cells, for example Vero cells, which technique can more than double blastocyst formation rates (Ménézo *et al.*, 1990; Plachot *et al.*, 1995). There have been a number of studies using these co-culture techniques which
5 have shown increased implantation rates after blastocyst transfer (Ménézo *et al.*, 1992), particularly in women with repeated previous implantation failures (Oliveness *et al.*, 1955; Plachot *et al.*, 1955).

Co-culture is time consuming and expensive and concerns have been expressed about
10 possible transfer of disease from contaminated cultures (Oliveness *et al.*, 1955), in particular there is a concern relating to viral contamination which contamination is considered to be virtually impossible to fully eliminate. A safer and more practical approach is to attempt to produce a culture medium able to sustain embryo development through to the blastocyst stage that is independent of co-culture.

15 One approach to enhance *in vitro* embryo development without using co-culture techniques is to attempt to define factors that might be used to enhance embryo development in *in vitro* culture. A number of attempts have been already made to identify factors that might assist and amongst the promising factors are various stimulatory factors known as cytokines.
20 One such factor, leukaemia inhibitory factor (LIF) has already been indicated as being positive in this regard for humans (Dunlison *et al.* 1996) and livestock species, US Patent specification 5418159.

25 One of the many factors also currently under investigation in both animals and humans relative to conception and embryo development is granulocyte-macrophage colony-stimulating factor (GM-CSF). However to date there has been no definite indication that a medium supplemented with GM-CSF would be sufficient to enhance the *in vitro* development of embryos to the blastocyst stage in a defined culture medium.

30 GM-CSF is a 23-29 kD glycoprotein which although secreted in a soluble form *in vitro*, is one of many cytokines known to be sequestered and immobilised in the ECM (extracellular matrix) *in vivo* through association with heparan sulphate. GM-CSF was originally characterised as a hemopoietic regulator and determinant of the maturation and behaviour of myeloid leukocytes in peripheral tissues. It is now known that GM-CSF is produced by
35 a diversity of cell types including T-lymphocytes, monocytes, macrophages, fibroblasts, endothelial cells and epithelial cells.

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The uterine epithelium has been identified by *in situ* hybridisation and in *in vitro* cell isolation studies as a major source of GM-CSF in the mouse uterus (Robertson *et al* 1992, Robertson *et al* 1994) and human oviduct and uterus (Zhao and Chegini 1994, Giacomini *et al* 1995). A role for GM-CSF in reproductive processes was supported by studies
5 perturbing the cytokine environment during early pregnancy *in vivo* (Tartakovsky and BenYair, 1991) and experiments showing impaired fertility in genetically GM-CSF deficient mice (Robertson *et al* 1999).

Studies of radio-labelled ligand binding show clearly that murine blastocysts bind ^{125}I -
10 GM-CSF specifically, indicating that they express at least the low affinity form of the GM-CSF receptor. This conclusion was supported by RT-PCR analysis, which showed that blastocysts express mRNA for the α -subunit of the GM-CSF receptor complex. A similar situation was found to exist in human embryos. GM-CSF-R was expressed at similar
15 levels through the first four days of murine and human embryo development, from fertilisation to blastocyst stage. However mRNA for the β -subunit of the GM-CSF receptor complex was not detected in embryos of either species by the RT-PCR technique. Together, these data suggest that embryos express GM-CSF receptor from at least as early
20 as fertilisation, but that it may be of the low affinity form. The embryo therefore falls into the same category as endothelial cells and other non-hemopoietic cells which exhibit a biological response to GM-CSF despite expressing only low affinity receptors. Although
25 it seems clear in hemopoietic cells that the α -subunit of the GM-CSF receptor cannot on its own transduce proliferative signal, it is not known whether the α -subunit can in some circumstances initiate responses in cells in the absence of the β -subunit. The recent discovery of unconventional forms of the GM-CSF receptor in the human suggests that
this may be possible.

It has also been shown that binding of cognate ligands to the GM-CSF receptor α subunit in isolation may mediate increased glucose transport via a phosphorylation-independent pathway (Ding *et al.*, 1994). Recent experiments by the inventor show that culture with
30 recombinant mouse GM-CSF (mGM-CSF) stimulates increased glucose uptake in murine blastocysts, to an extent achievable with known glucose transport stimulants such as insulin-like growth factor-1, suggesting that this cytokine may stimulate metabolism in murine embryos.

35 There is some evidence to indicate that GM-CSF also participates in regulation of embryonic growth. Conditioned media rich in mGM-CSF have been found to be effective particularly in promoting blastocyst development, particularly in the attachment of hatched blastocysts to serum attachment factors in plastic culture dishes (Robertson *et al.*, 1991).

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The media was conditioned by cells from LPS activated mouse lung tissue, and contains a number of other factors which could contribute to the embryotrophic activity.

5 In further studies by the inventor one cell and eight cell mouse embryos were cultured with or without recombinant mouse GM-CSF (rm GM-CSF) in a defined medium, and again there was a significant increase in the rate at which hatched blastocysts attached to the culture dish. The proportion of embryos developing to eight cell or blastocyst stage was not altered by cytokine. The rate at which embryos developed to blastocysts and hatched from the zona pellucida was also similar, regardless of whether cytokine was present or
10 absent.

In further experiments the survival and/or proliferation of blastomeres within developing mouse blastocysts, particularly inner mass cells, was shown to be enhanced by exposure to native GM-CSF *in vivo*, or by recombinant GM-CSF *in vitro* (Robertson *et al.*, 1998).
15

Several groups have reported both positive and negative effects of GM-CSF on various stages of early embryo development. Hill *et al.* (1987) have found that GM-CSF at high doses (> 1000 U/ ml) inhibited the development of 2-cell embryos into morulae. In two studies, ectoplacental cone trophoblast has been found to proliferate in response to GM-CSF (Armstrong and Chaouat 1989; Lea and Clark 1993), but in the second instance an effect was obtained with native but not recombinant cytokine. Haimovoci *et al.* (1991) found that 250 U/ ml or more of GM-CSF inhibited the attachment of blastocysts to fibronectin-coated culture dishes in the absence of serum. Lea and Clark (1993) have reported that recombinant GM-CSF (at between 10 and 100 U/ ml) inhibited the
20 incorporation of ³H-thymidine into outgrowing, implanted blastocysts, in a dose dependant manner. Tartakovsky and Ben-Yair (1991) found that systemic GM-CSF administration markedly enhanced early embryonic development *in vivo*, but did not note any effect of GM-CSF on embryonic development *in vitro*. These results are difficult to reconcile. However, the differences are likely to be related to the developmental stages
25 examined, the methods for embryo culture, the strains of mice, and the sources and concentrations of cytokine used. For example, some cytokine preparations may contain potentially embryotoxic contaminants such as endotoxin. In addition, there is emerging evidence that there may be more than one mechanism by which GM-CSF is able to exert its effects in target cells, and it is possible that the glycosylation state of the cytokine
30 (which would also be dependant upon its source) may be important for binding to unconventional receptors.
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A study of bovine embryos (de Moraes and Hansen 1997) used recombinant bovine GM-CSF (rbGM-CSF) in attempt to enhance embryo development to blastocyst stage. The rbGM-CSF only had a significant impact on the proportion of embryos developing to blastocyst stage at very high levels of 10 ng/ml, and the numbers of embryos tested were relatively low so the results might be viewed with some concern. Additionally it was found however that the proportion of blastocysts that expanded or hatch dropped significantly with the 10 ng/ml rbGM-CSF and 1 ng/ml rbGM-CSF and thus can be seen an adverse effect on the capacity of the blastocysts to be used subsequently as their development had essentially terminated *in vitro*.

10

SUMMARY OF THE INVENTION

The present invention results from a finding that recombinant human GM-CSF (rhGM-CSF) is effective at substantially increasing the proportion of early embryos that develop to blastocyst and increasing the proportion of those embryos that continue to expanded blastocyst and then hatched blastocyst stages of development. The net result is that a much greater proportion of embryos can now be grown to blastocyst stage and used for implantation in an IVF program in humans.

This contrasts with the mixed findings in other species, whereby only moderate and inconsistent effects on development to blastocyst stage and beyond were reported.

This finding has implications in the formulation of media for use in *in vitro* culturing of embryos to blastocyst stage and in methodologies of growing such embryos and in the manner in which IVF programs are conducted. It is anticipated that this invention will lead to a greater success rate in such IVF programs.

Thus in one broad form of a first aspect the invention could be said to reside in a medium for propagation of early stage embryos to blastocyst stage, said medium containing an effective amount of human GM-CSF to increase the percentage of pre-blastocyst embryos which develop to transfer ready blastocysts.

Transfer ready blastocysts are embryos developed to the stage where a blastocoel cavity is clearly evident and comprises greater than 50% of the volume of the embryo. This stage would in the *in vivo* situation normally be achieved 4-5 days after fertilisation, soon after the embryo has traversed to fallopian tube and arrives in the uterus.

In one form the medium is a serum deprived medium. The serum deprived medium is desirable in so far as the risk of contamination is drastically reduced. The term serum

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deprived when used in this specification refers to a medium that does not include serum, or any partially defined serum fraction as an additive, but may include a medium that includes serum derived components that have been substantially purified from serum, and may or may not have been modified.

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In another form the medium might be a fully defined medium.

Most preferably the human GM-CSF is in purified form, and most preferably purified in from a non-animal and non-human source, and might thus be purified from a recombinant micro-organism.

10

The GM-CSF receptors of embryos appears to be somewhat unique in composition compared to GM-CSF receptors elsewhere and it is therefore likely that the support for embryo growth may not require a fully native GM-CSF. The hGM-CSF may thus be modified or altered in any one of a number of ways and may or may not need to be glycosylated. The hGM-CSF may be truncated, include amino acid deletions and substitutions or may be a recombinant molecule with another growth factor such as perhaps LIF.

15

Where rhGM-CSF is used it is anticipated that the level of rhGM-CSF in the medium as used will be approximately 1 ng/ml which a physiologically normal level. However, ranges of concentration are also possible and it is anticipated that concentrations ranging from about 0.01 ng/ml to about 5 ng/ml will also give an increase depending on the specific activity of the recombinant or native GM-CSF preparation. It will be understood however that it might be found that concentrations outside of this might also lead to a beneficial effect.

20

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A base medium to which the hGM-CSF is added might be any one known to the person skilled in the art.

30

The medium in which this invention might be used can be any medium suitable for use for the *in vitro* support of embryo development and growth. A suitable medium might include but is not limited to HTF medium (Quinn, 1985a), Modified Whittens medium (Trounson, 1984), Whittinghams T6 medium (Trounson, 1984), Hams F10 (Trounson *et al.*, 1982a), Earles solution (Edwards and Purdy, 1982), IVF50 (Scandinavian IVF Science), S2 (Scandinavian IVF Science), G1.2 (Scandinavian IVF Science) and G2.2 (Scandinavian IVF Science) which references are incorporated herein by references in relation to the media.

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In a broad form of a second aspect, the invention could be said to reside in a method of growing early stage human embryos to transfer ready blastocysts, including the step of incubating the embryos *in vitro* in a culture medium containing an effective amount or

5 human GM-CSF for a time and under conditions to increase the proportion of transfer ready blastocysts.

It is anticipated that the early stage embryos will generally be contacted with GM-CSF at an early stage. The early stage of the embryos may be from immediately after fertilisation,

10 through to several days after fertilisation but preferably before 4 days. Most preferably the contact will be within 2 days of fertilisation. It will be understood that these will be at the 2-16 cell, morula or pre-blastocyst. Generally a 1 day embryo will have 2 cells, 3 day is 16 cell or morula and in 4 to 5 days will develop to a blastocyst. A blastocyst is characterised by a clearly visible blastocoel cavity.

15

It is anticipated that the *in vitro* growth will be continued until the blastocysts reach the day 5 to 6 stage, however, in certain embodiments of the invention the culturing of the embryo may be to an earlier, or later stage.

20 In one form this second aspect of the invention comprises culturing of the embryo in a serum deprived medium including human GM-CSF until blastocyst stage is reached, and then transferring to a second medium including human GM-CSF for further culturing.

In a broad form of a third aspect the invention could be said to reside in an IVF program comprising the steps of:

25

- contacting an human egg with a human sperm to form a conceptus
- growing the resulting conceptus at least after the 8 cell stage embryo has formed *in vitro* in a defined culture medium containing an effective amount of human GM-CSF for a time and under conditions to increase the chance of achieving a transfer ready blastocyst
- 30 - transferring the transfer ready blastocyst into a compatible human uterus

For a better understanding, the invention will now be described with reference to a number of examples.

35 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Effect of GM-CSF on development of embryos to blastocysts, according to embryo grade. Data is the number of embryos

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developed to blastocyst from experiments 1, 2 and 3 combined, expressed as a percentage of the initial number of cleaved (2-4 cell) embryos. The number of embryos in each group are given in parentheses.

5

Figure 2: The effect of GM-CSF on the development of embryos to blastocyst, hatching and attachment stages. Data is the number of embryos developed to or beyond each stage, from experiments 1,2 and 3 combined, expressed as a percentage of the initial number of cleaved (2-4 cell) embryos. 2-C = 2-cell embryos; 8-C = 8-cell embryos; M = morulla; B = blastocyst; Exp B = expanded blastocyst; H = hatching; A = attached with trophectoderm outgrowth.

10

Figure 3: The effect of GM-CSF on the rate of development of embryos to blastocyst. Data is the number of blastocysts at each time point, from experiments 1,2 and 3 combined, expressed as a percentage of the total number of blastocysts at 144 h post insemination.

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Figure 4 RT-PCR analysis of GM-CSF receptor mRNA expression in human blastocysts. Total cellular RNA was extracted from TF-1 cells and each of two cohorts of blastocysts (B ϕ 1 and B ϕ 2), reverse transcribed by random priming and amplified by PCR with GM-R α , GM-R β or actin-specific primers using conditions listed in Table 7.

20

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Figure 5 The effect of GM-CSF on the rate of development of embryos to the blastocyst stage; (A) an early blastocyst (day 5, 112 h post-insemination) from the control group; (B) an expanded blastocyst (day 5, 112 h post-insemination) cultured in rhGM-CSF; (C) a fully hatched blastocyst attached to the culture dish (day 6, 144 h post-insemination); (D) an attached blastocyst cultured in rhGM-CSF showing trophectoderm outgrowth (arrow; day 8, 200 h post-insemination).

30

35

Figure 6 The effect of GM-CSF on the number of total cells (TCN), inner cell mass (ICM) cells and trophectoderm (TE) cells in day 5 blastocysts (120-124 h post-insemination). Values are mean \pm SD of blastocysts

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cultured in 2 ng / ml rhGM-CSF (n=11) and blastocysts cultured in media alone (n=10).

DETAILED DESCRIPTION OF THE INVENTION

5

EXAMPLE 1.

Measurement of embryonic viability and development

Materials and Methods

10 The embryos used in this study were donated by couples undergoing IVF treatment at Fertilitetscentrum AB, Göteborg, Sweden. Embryos frozen at the 2-4 cell stage were thawed at or beyond their one year storage limit in liquid nitrogen. Ethics approval for the study was obtained from the research ethics committee at University of Göteborg (number 700-96).

15

Ovarian stimulation and *in vitro* fertilisation

Patients received 300 µg buserelin gonadotrophin-releasing hormone agonist (GnRHa; Suprecur; Hoechst, Frankfurt, Germany) three times daily intranasally, starting 1 week before expected menses and lasting for two weeks. Down-regulation was confirmed by a
20 serum estradiol content of <0.2nmol/l. Patients were then given recombinant follicle stimulating hormone (r-FSH; Gonal-F; Serono Laboratories, Aubonne, Switzerland; 150-225 IU / day sub-cutaneously). The starting dose was dependent on the patient age and/or previous response during ovarian stimulation (Wikland *et al.*, 1994). The ovarian response was monitored by ultrasound and serum estradiol levels as previously described (Bergh *et al.*, 1997). GnRHa and rFSH were administered until there was at least one follicle >18
25 mm in mean diameter and two others >16 mm. Finally, oocyte maturation was triggered by one sub-cutaneous injection of 10 000 IU of hCG (Profasi; Serono Laboratories).

Oocytes were retrieved 36-38h after hCG administration, assessed morphologically and
30 fertilised *in vitro*. The embryos were cultured in IVF-50 (Scandinavian IVF Science AB, Göteborg, Sweden) and frozen on day 2 using a 3-step propanediol cryo-preservation kit (Freeze Kit 1, Scandinavian IVF Science) according to the manufacturers instructions.

Recombinant GM-CSF

35 Recombinant human (rh)GM-CSF was obtained from R&D Systems Europe Ltd, Oxon, UK. The biological activity of the recombinant cytokine preparations was measured in a bioassay employing a GM-CSF responsive cell line (human myeloid TF-1 cell line), essentially as described by (Kitamura *et al.* 1989). Duplicate serial 1:2 dilutions were

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incubated with 2000 TF-1 cells in 200 μ l of RPMI-1640 (Gibco) supplemented with 10% fetal calf serum (FCS; Commonwealth Serum Laboratories, Australia), 5×10^{-5} M β -mercaptoethanol and antibiotics. After 2 days, cultures were pulsed with 1 μ Ci of 3 H thymidine (Amersham, Arlington Heights, IL) for 6 hours, harvested onto glass fibre paper using a Titretech automated cell harvester and radioactivity measured in a liquid scintillation beta counter.

Embryo thawing, allocation and culture

Frozen 2-4 cell embryos were thawed in four steps using a propanediol method for embryo thawing (Thaw Kit 1, Scandinavian IVF Science) following instructions given by the manufacturer. The viable embryos were classified and graded according to criteria listed in Table 1.

Table 1: Embryo classification criteria

Embryo Grade	Morphology
A	Regular blastomeres without fragments
B	Regular or irregular blastomeres, up to 30% fragments
C	Regular or irregular blastomeres, more than 30% fragments
D	50% of the blastomeres dead after thawing

To avoid bias the embryos were randomly allocated, with regard to patient and embryo grade, into the different culture groups (Table 2). The embryos were cultured in groups of five embryos per drop. To avoid the toxic effects of ammonium, released due to metabolism and breakdown of amino acids, the culture media was renewed every 48 h until hatching occurred. In two experiments the embryos were cultured in 20 μ l drops of IVF-50 (Scandinavian IVF Science) containing 2 ng/ml rhGM-CSF (diluted 1:25 000 from stock material) or carrier (2 ng/ml BSA, diluted 1:1 000 from stock material). Culture drops were covered by 4 ml Ovulil-200 (Scandinavian IVF Science) in Falcon 3004 dishes (Becton-Dickinson Labware, Franklin Lakes; NJ, USA). When blastocysts were detected these were transferred into 1 ml of S2 (Scandinavian IVF Science) in Falcon 3037 dishes, containing 5% FCS and 2 ng/ml rhGM-CSF or carrier. Developmental stage was scored every 8h from thawing until 2300 h on day 8 (200 h post-insemination).

In a third experiment the embryos were transferred from IVF-50 into S2 medium (Scandinavian IVF Science) at the 6-8 cell stage. Additions of GM-CSF and carrier were the same as in the two previous experiments. When blastocysts were detected they were

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transferred to Falcon 3037 dishes, coated 24 h previously with Biomatrix EHS (Boehringer Ingelheim Bioproducts, Heidelberg, Germany). Developmental stage was scored every 8 h from thawing until 2300 h on day 8 (200 h post-insemination). Embryo scoring in each of the experiments was performed by the same person (CS).

5

Statistical analysis was performed using Fisher's exact test and independent samples t-test (StatSoft, Inc.). Differences in data were considered significant when $P < 0.05$.

10

Table 2: Distribution of grades amongst thawed 2-4 cell embryos

Embryo grade	Control (%)	GM-CSF (%)
A	20	20
B	22	27
C	32	33
D	26	20
N	50	49

Grades are defined in Table 1.

Results

- 15 The rate and extent of development of 2-4 cell embryos to the blastocyst and hatching blastocyst stages was significantly increased by the addition of rhGM-CSF to culture medium (Table 3).

20 **Table 3: Rate and extent of embryo development in the presence or absence of rhGM-CSF**

	n	%BΦ	T ₅₀	%H	N	% BΦ	T ₅₀	%H
Expt	Control				RhGM-CSF			
1	16	38	122	50	15	60	121	89
2	16	38	116	50	16	81	98	100
3	18	17	127	33	18	83	105	53
Total	50	31	122	47	49	76 ^a	108 ^b	78 ^c

%BΦ= % of viable thawed 2-4 cells reaching blastocyst stage. ^a $p < 0.0001$

T₅₀ = number of hours post-insemination at which 50% blastocysts develop.

^b $p = 0.0002$ at 112h PI

- 25 %H = % of blastocysts which fully or partially hatch. ^c $p = 0.009$

A comparison between the proportion of embryos reaching blastocyst stage and beyond in experiment 1 and 2 (culture media containing 5% FCS from day 5) and experiment 3 (serum-free media) are presented in Table 4. There are no significant differences between

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the two groups, showing that the beneficial effect of GM-CSF is not dependent on the presence of FCS.

5 **Table 4:** Percent embryos developing up to or beyond each developmental stage in experiment 1 and 2 (FCS added) compared with experiment 3 (no FCS added).

	N	% BΦ	% Exp BΦ	% Hatching	Attached
Control (exp 1+2)	32	37	28	19	0
GM-CSF (exp 1+2)	31	71	68	68	38
Control (exp 3)	18	17	6	6	0
GM-CSF (exp 3)	18	83	61	44	22

BΦ= blastocyst; Exp BΦ= expanded blastocyst

10 Although fewer poor quality embryos (grades C & D) reach blastocyst stage than good quality (grades A & B), GM-CSF exerted a comparable effect in all groups, with similar or slightly higher increases in the proportion of poor compared with good quality embryos achieving blastocyst stage (Fig. 1).

15 The majority of embryos grown in media alone were lost at the 4-16 cell stage. The beneficial effect of GM-CSF on blastocyst development appeared to result from rescue of this loss, with an 80% increase in the numbers of embryos reaching the morula stage of development (Fig. 2). Furthermore, the developmental potential of blastocysts was increased by culture in GM-CSF, since the rate of hatching was greater for embryos grown in GM-CSF. Similarly, blastocysts grown in GM-CSF (15/29), but not in control media (0/15), attached to the culture dish and showed trophoblast outgrowth (Fig. 2 and 20 Fig. 5).

Finally, embryos cultured in the presence of rhGM-CSF had a significantly higher rate of development, with 50% blastocyst development achieved 14 hours earlier in GM-CSF compared with the control group (Fig. 3).

25

Conclusions

These results support the hypothesis that GM-CSF secreted into the female reproductive tract during early pregnancy promotes embryo growth and development. The addition of GM-CSF to culture media promotes the formation of blastocysts even with poor post thaw 30 quality embryos. Our results also show a beneficial effect of GM-CSF on blastocyst expansion, hatching, attachment and trophectoderm outgrowth. Although the functional significance of hatching *in vitro* is unknown, blastocyst expansion is one of the best criteria for blastocyst viability and developmental potential.

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The cleavage rate of embryos is suggested to be an indicator of embryo quality (Shoukir *et al.*, 1997), and the rate of embryo development is known to be higher *in vivo*.

Importantly, development of embryos to blastocysts was achieved significantly faster in
5 the presence of rhGM-CSF.

EXAMPLE 2

Measurement of embryonic viability and development - variation of media and source of GM-CSF

10 *Materials and Methods*

The embryos used in this study were donated by couples, after ovarian stimulation and *in vitro* fertilisation, as described in Example 1. For culture experiments, embryos frozen at the 2-4 cell stage were thawed at or beyond their one year storage limit in liquid nitrogen.

15 The blastocysts used for the differential staining experiment were cultured from excess embryos, surplus to treatment and freezing requirements.

Recombinant GM-CSF

Two different commercial sources of recombinant human (rh)GM-CSF were used in these
20 experiments. A laboratory grade preparation was obtained from R&D Systems Europe Ltd, Oxon, UK, and a pharmaceutical grade preparation, Molgramostim (Leucomax) was obtained from Schering & Plough, Madison, NJ, USA. The biological activity of both recombinant cytokine preparations were measured in a bioassay employing a GM-CSF responsive cell line (human myeloid TF-1 cell line), as described in Example 1.

25

Embryo thawing, allocation and culture

Frozen 2-4 cell embryos were thawed and allocated randomly to experimental groups as as described in Example 1. Embryo culture was performed as described in Example 1, in two different sequential media systems using two different commercial sources of rhGM-
30 CSF. After thawing, the embryos were cultured first in G1.2 (Scandinavian IVF Science) or IVF-50. At 6-8 cell stage the embryos were transferred into G2.2 (Scandinavian IVF Science) or S2. The experiment included 6 groups: (a) G1.2/G2.2 alone, (b) G1.2/G2.2 containing 2 ng/ml rhGM-CSF (R&D Systems) (c) G1.2/G2.2 containing 2 ng/ml Molgramostim (Schering & Plough; diluted 1:75 000 from stock material), (d) IVF-50/S2
35 alone, (e) IVF-50/S2 containing 2 ng/ml rhGM-CSF (R&D Systems) (f) IVF-50/S2 containing 2 ng/ml Molgramostim. Developmental rate was scored every eighth hour until expanded blastocyst stage. Blastocysts were scored on day 5 at 120 h post-insemination according to criteria described previously (Dokras *et al.*, 1993). Briefly, grade A

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blastocysts exhibited an expanded cavity with a distinct trophectoderm (TE) and an eccentrically located inner cell mass (ICM); grade B blastocysts were not yet expanded but otherwise morphologically identical to A; and grade C blastocysts exhibited poor morphology characterised by a number of degenerative foci in the ICM and TE and a poorly developed blastocoel cavity. Embryo scoring in each of the experiments was performed by the same person (CS).

Statistical analysis was performed using Fisher's exact test and independent samples t-test (StatSoft, Inc.). Differences in data were considered significant when $P < 0.05$.

Differential labelling of blastocysts

Differential labelling was performed using a modification of a protocol described previously (Handyside and Hunter, 1984). Human blastocysts were cultured from excess embryos, surplus to treatment and freezing. On day 5 of culture (120 - 128 h post-insemination) the zona was removed in Acid Tyrodes solution containing 4 mg/ml PVP (360 000 Mw) and embryos were washed once in Gamete-100 (Scandinavian IVF Science) and three times in albumin-free S2 containing 4 mg/ml PVP (S2-PVP). The blastocysts were incubated in trinitro-benzene sulfonic acid (TNBS, Sigma Chemical Co., St Louis, MO, USA; 10 mM in S2-PVP pH 8.5, 4°C / 20 min in the dark) and washed three times in Gamete-100. TNBS-treated blastocysts were incubated in anti-dinitro-phenyl antibody (anti-DNP; Sigma, 0.2 mg/ml diluted in Gamete-100; 37°C / 30 min) Embryos were then washed and incubated in guinea pig complement serum (Sigma; diluted 1:10 in Gamete-100; 37°C / 30 min). Embryos were washed again and labelled with flouorochromes (Sigma; 0.05 mM bisbenzimidazole and 10 ug/ml propidium iodide in Gamete-100, 37°C / 30 min). After extensive washing embryos were fixed briefly in 1% paraformaldehyde and 0.5% glutaraldehyde in PBS, mounted under cover-slips in 20% glycerol in PBS and examined by fluorescence microscopy using a 400 nm exitation filter. Nuclei stained pink were scored as lysed trophectoderm cells (TE) and blue nuclei were scored as viable inner cell mass cells (ICM).

Results

This experiment demonstrates the effect of culture media and source of recombinant cytokine on GM-CSF stimulated blastocyst development. Cytokine formulations in two different sequential culture media systems were found to have equivalent bioactivities in the TF-1 cell proliferation assay (data not shown). There were no significant differences between the blastulation rates achieved in the two different culture media systems (Table 5). Both the rate and extent of development of 2-4 cell embryos to blastocysts was significantly increased by the addition of 2 ng/ml rh GM-CSF. The effect was comparable

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in extent in both G1.2/G2.2 and IVF-50/S2 sequential media combinations. Furthermore, the improvement in blastocyst development was achieved irrespective of the formulation of recombinant cytokine. The results also show that although culture in rhGM-CSF gives rise to more blastocysts, the distribution in morphological grade was comparable in treatment and control groups (Table 5).

Table 5: The effect of culture media and source of recombinant cytokine on GM-CSF stimulated blastocyst development.

	N	% B ϕ	A/B/C (%)
G1.2/G2.2 alone	23	30	57/29/14
G1.2/G2.2 + rhGM-CSF (R&D)	21	71**	67/20/13
G1.2/G2 + Molgramostim	19	63*	67/17/17
IVF-50/S2 alone	38	37	57/29/14
IVF-50/S2 + rhGM-CSF (R&D)	38	79***	67/26/7
IVF-50/S2 + Molgramostim	20	65*	70/15/15

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

The effect of culture in GM-CSF on blastomere number and allocation.

To investigate the effect of culture with GM-CSF on cell number and allocation to inner cell mass and trophectoderm cell lineage, blastocysts cultured with and without rhGM-CSF were analysed by immunosurgery and differential staining. Blastocysts cultured in the presence of rhGM-CSF had a significantly higher total cell number compared to blastocysts cultured in media alone (Fig. 6). An increase in the number of trophectoderm cells, and particularly in the number of inner cell mass cells, each contributed to the greater cell number in GM-CSF stimulated blastocysts.

EXAMPLE 3

IVF Media

The techniques and media used for embryo culture in IVF procedures have not changed a great deal since the 1980s. These procedures are set out most particularly in Kerin *et al* (1983), Trouson *et al* (1980), Trouson *et al* (1982), and Quinn *et al* (1985) which references are incorporated herein by references in relation to those procedures.

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The media in which this invention might be used can be any media suitable for use for the *in vitro* support of embryo development and growth. These media might include but are not limited to HTF medium (Quinn, 1985a), Modified Whittens medium (Trounson, 1984), whittinghams T6 medium (Trounson, 1984), Hams F10 (Trounson *et al*, 1982a),
5 Earles solution (Edwards and Purdy, 1982), IVF50 (Scandinavian IVF Science), S2 (Scandinavian IVF Science), G1.2 (Scandinavian IVF Science) and G2.2 (Scandinavian IVF Science) which references are incorporated herein by references in relation to the media.

10 EXAMPLE 4

Method of IVF treatment

IVF procedures have not changed a great deal since the 1980s. The procedures for IVF treatment used in this invention are standard procedures that are set out most particularly in
15 Kerin *et al* (1983), Trouson *et al* (1980), Trouson *et al* (1982), and Quinn *et al* (1985) which references are incorporated herein by references in relation to those procedures.

EXAMPLE 5

20 *The expression of GM-CSF receptors by human pre-implantation embryos in vitro**Material and Methods*

The embryos used in this study were donated by couples, after ovarian stimulation and *in vitro* fertilisation, as described in Example 1. Excess human 2-4 cell embryos surplus to
25 patients' requirements were cultured in 20 ml droplets of IVF-50 overlayed with paraffin oil. On day 3 (72 h post insemination) the embryos were transferred to S2. Embryos were collected at blastocyst stage of development. The embryos were washed in PBS, snap frozen in liquid nitrogen and stored at -70°C prior to RNA extraction.

30 Total cellular RNA was extracted from human GM-CSF responsive myeloid cells (TF-1 cell line), and from two cohorts each of twenty blastocysts using a method described by (Arcellana-Panlilio & Schultz, 1993). Residual chromosomal DNA was removed by treatment with RNase-free DNase (Boehringer Mannheim) for 60 min at 37°C. First strand cDNA synthesis was achieved by reverse transcription (RT) of RNA primed with
35 random hexamers using a Superscript RNase H-reverse transcriptase kit (Gibco) essentially according to the manufacturer's instructions. Detection of mRNA by RT-PCR was performed using primer pairs specific for the α -chain and β -chain of the GM-CSF receptor (GM-R α and GM-R β), and β -actin (detailed in Table 7) and reagents supplied in

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receptor (GM-R α and GM-R β), and β -actin (detailed in Table 7) and reagents supplied in a Taq DNA polymerase kit (Biotech International Ltd., Perth), essentially as described previously. The number of cycles and annealing temperature used for each primer pair are also given in Table 7. To increase the sensitivity of the GM-R β PCR, a nested primer design was employed, wherein cDNA was amplified by 30 cycles with GM-Rb 'external' primers followed by 25 cycles with GM-R β 'internal' primers. Each PCR product was analysed by electrophoresis through a 2% agarose gel containing EtBr, and visualised by trans-illumination with UV-light. Gels were photographed and the size of the PCR products was verified by comparison of their relative mobility to molecular weight markers.

Table 7. Primer sequences

Target	Genebank accession number	Primer sequence	Position in sequence	Cycle number / annealing temp	Product size
β -actin	M12481	5' tgtgatgggtgggtatgggtc 3' tagatgggcacagtgtgggt	48-67 400-419	35 / 62°C	372 bp
GM-R α	M64445	5' catgcttctcctggtgacaa 3' gtgactccttcacatgcagaca	162-181 421-440	40 / 60°C	279 bp
GM-R β	M59941 / M38275	external: 5' ctacaccagccacatcacct 3' agtcctgaagccgctttag internal: 5' gagccagtgtcctgtgacct 3' tggcctgggtcggtgctgat	142-161 550-569 239-258 449-468	30 / 65°C 25 / 65°C	428 bp 230 bp

Results

The expression of GM-CSF receptor expression by *in vitro* generated blastocysts was examined by RT-PCR. Each of two preparations of blastocysts were found to express mRNA for the α -chain of the GM-CSF receptor, but mRNA for the β -chain was not detected, even when a highly sensitive nested PCR protocol was used (Fig. 4).

Conclusions

Expression of GM-CSF receptor α -chain mRNA was detected in each of two blastocyst cDNA preparations. These results indicate that human blastocysts have the molecular capacity to bind and respond to GM-CSF. The expression of the α -subunit in the absence of the β -chain may benefit blastocyst glucose transport and thus optimise the culture environment. Increased glucose uptake is likely to promote blastomere metabolic activity, and hence cell division, and may also prolong cell survival through the prevention of apoptosis.

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CLAIMS

1. A medium for propagation of early stage embryos to blastocyst stage, said medium containing an effective amount of human GM-CSF to increase the percentage of pre-
5 blastocyst embryos which develop to transfer ready blastocysts.
2. A medium for propagation of early stage embryos to blastocyst stage according to claim 1 wherein the human GM-CSF is in purified form.
- 10 3. A medium for propagation of early stage embryos to blastocyst stage according to claim 2 wherein the human GM-CSF is purified from a non-animal and non-human source.
4. A medium for propagation of early stage embryos to blastocyst stage according to
15 claim 3 wherein the human GM-CSF is purified from a recombinant micro-organism.
5. A medium for propagation of early stage embryos to blastocyst stage according to claim 2 wherein the human GM-CSF is not fully native and is modified or altered.
- 20 6. A medium for propagation of early stage embryos to blastocyst stage according to claim 5 wherein the human GM-CSF is modified or altered in any one or more of the ways selected from the list including truncation, amino acid deletion, amino acid substitution, and a recombinant molecule with another growth factor.
- 25 7. A medium for propagation of early stage embryos to blastocyst stage according to claim 4 wherein the level of human GM-CSF in the medium is between 0.01 ng/ml and 5 ng/ml.
8. A medium for propagation of early stage embryos to blastocyst stage according to
30 claim 7 wherein the level of human GM-CSF in the medium is 0.01 ng/ml.
9. A medium for propagation of early stage embryos to blastocyst stage according to claim 7 wherein the level of human GM-CSF in the medium is 2 ng/ml.
- 35 10. A medium for propagation of early stage embryos to blastocyst stage according to claim 7 wherein the medium is a serum deprived medium.

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11. A medium for propagation of early stage embryos to blastocyst stage according to claim 10 wherein the serum deprived medium includes serum derived components that have been substantially purified from serum.
- 5 12. A medium for propagation of early stage embryos to blastocyst stage according to claim 7 wherein the medium is a fully defined medium.
- 10 13. A method of growing early stage human embryos to transfer ready blastocysts, the method including the step of incubating the embryos *in vitro* in a culture medium containing an effective amount of human GM-CSF for a time and under conditions to increase the proportion of transfer ready blastocysts.
14. A method of growing early stage human embryos to transfer ready blastocysts as in claim 13 wherein the early stage embryos are contacted with GM-CSF at an early stage.
- 15 15. A method of growing early stage human embryos to transfer ready blastocysts as in claim 14 wherein the early stage of the embryos is from immediately after fertilisation, through to several days after fertilisation.
- 20 16. A method of growing early stage human embryos to transfer ready blastocysts as in claim 15 wherein the early stage of the embryos is before 4 days.
17. A method of growing early stage human embryos to transfer ready blastocysts as in claim 16 wherein the contact is within 2 days of fertilisation.
- 25 18. A method of growing early stage human embryos to transfer ready blastocysts as in claim 15 wherein the *in vitro* growth is continued until the blastocysts reach the day 5 to 6 stage.
- 30 19. A method of growing early stage human embryos to transfer ready blastocysts as in claim 18 wherein the embryo is cultured in a serum deprived medium including human GM-CSF until blastocyst stage is reached, and then transferred to a second medium including human GM-CSF for further culturing.
- 35 20. A method of growing early stage human embryos to transfer ready blastocysts as in claim 19 wherein the human GM-CSF is in purified form.

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21. A method of growing early stage human embryos to transfer ready blastocysts as in claim 20 wherein the human GM-CSF is purified from a recombinant micro-organism.
- 5 22. A method of growing early stage human embryos to transfer ready blastocysts as in claim 21 wherein the level of human GM-CSF in the medium is between 0.01 ng/ml and 5 ng/ml.
- 10 23. An IVF program comprising the steps of:
- contacting an human egg with a human sperm to form a conceptus
- growing the resulting conceptus at least after the 8 cell stage embryo has formed *in vitro* in a defined culture medium containing an effective amount of human GM-CSF for a time and under conditions to increase the chance of achieving a transfer ready blastocyst
- transferring the transfer ready blastocyst into a compatible human uterus.
- 15 24. An IVF program as in claim 23 wherein the embryo is cultured in a serum deprived medium including human GM-CSF until blastocyst stage is reached, and then transferred to a second medium including human GM-CSF for further culturing.
- 20 25. An IVF program as in claim 24 wherein the human GM-CSF is in purified form.
26. An IVF program as in claim 25 wherein the human GM-CSF is purified from a recombinant micro-organism.
- 25 27. An IVF program as in claim 21 wherein the level of human GM-CSF in the medium is between 0.01 ng/ml and 5 ng/ml.

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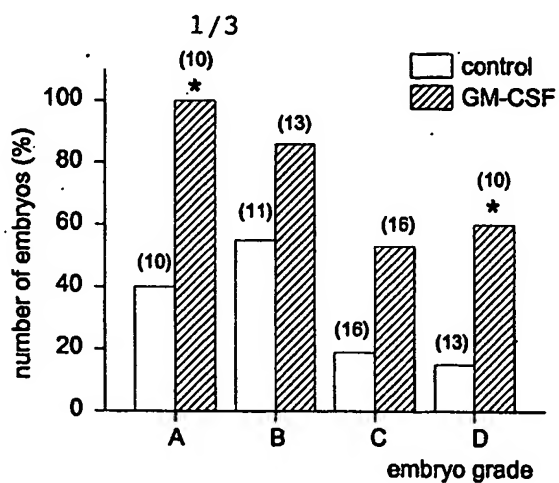


Figure 1

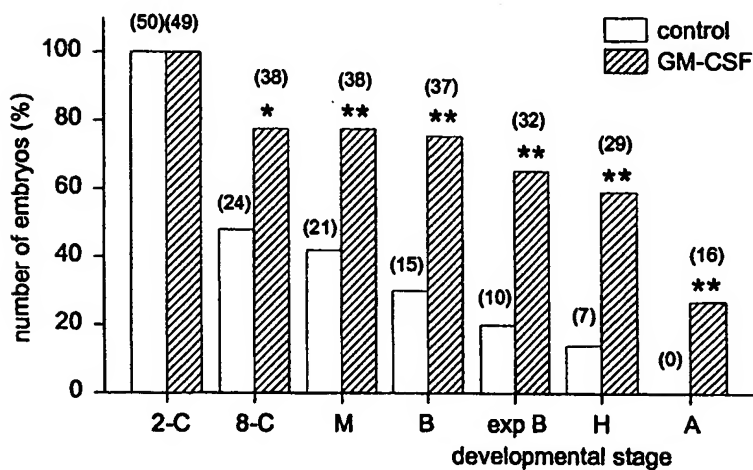


Figure 2

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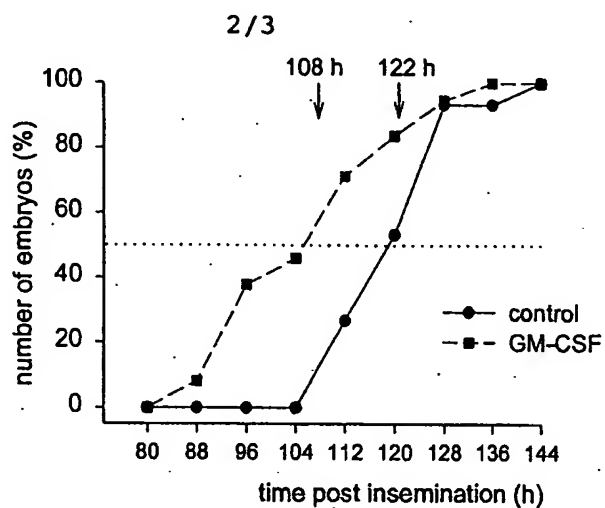


Figure 3

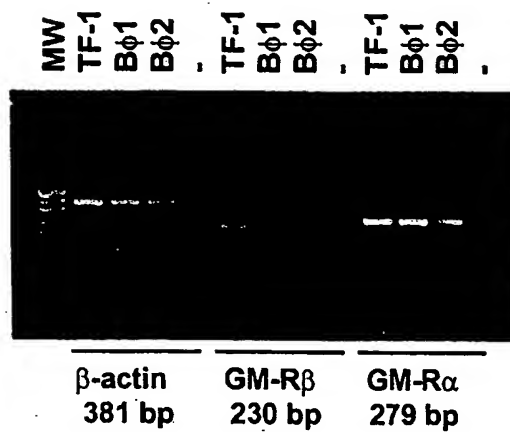


Figure 4

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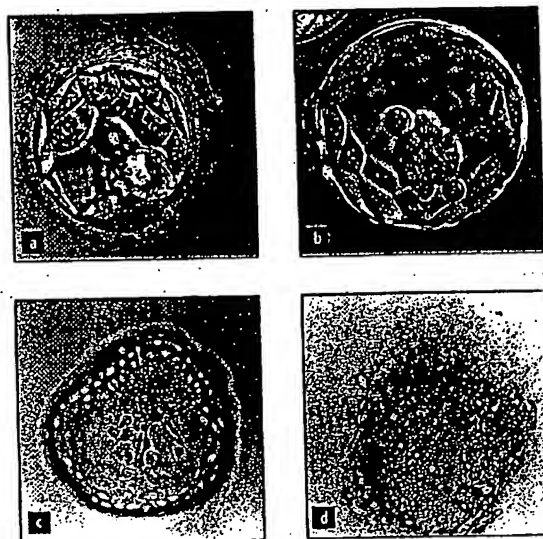


Figure 5

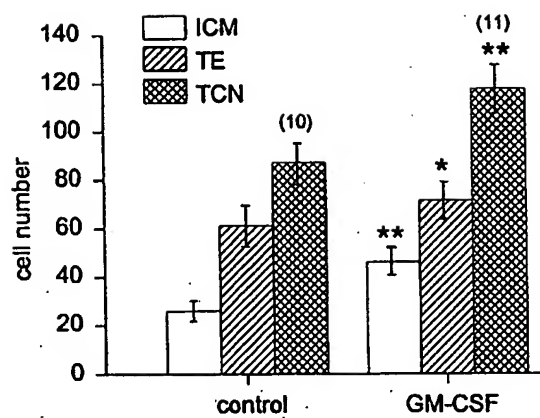


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00499

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁶: C12N 5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT AND CHEM ABS Key words used (kw) see electronic data base box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE, JAPIO, USPM Key words used (kw) see electronic data base box below.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, USPM: Granulocyte macrophage colony stimulating factor and (pregnan: or fertil: or embryo: or blastocyte#)
CA, Medline: Granulocyte macrophage colony stimulating factor and (blastocyst? or blastoderm? or trophoblast?)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. A. S. de Moraes and P. J. Hansen "Granulocyte-Macrophage Colony-Stimulating Factor promotes development of <i>in vitro</i> produced bovine embryos" Biology of Reproduction 57, 1060-1065 (1997) See whole document.	1-27
X	F. Haimovici <i>et al</i> "The effects of soluble products of activated lymphocytes and macrophages on blastocyst implantation events <i>in vitro</i> " Biology of Reproduction 44, 69-75 (1991) See whole document.	1-27
Y	P. P. Jokhi <i>et al</i> "Production of granulocyte-macrophage colony-stimulating factor by human trophoblast cells and by decidual large granular lymphocytes" Human Reproduction volume 9, 1660-1669 (1994) See "Introduction" on page 1660.	1-5, 13-21

☒ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"T" later document published after the international filing date Or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one Or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
13 August 1999

Date of mailing of the international search report
16 AUG 1999

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INTERNATIONAL SEARCH REPORT

International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Martal <i>et al</i> "Recent developments and potentialities for reducing embryo mortality in ruminants: the role of IFN- τ and other cytokines in early pregnancy" <i>Reproduction, fertility and development</i> 1997, 9 355-380. See page 362.	1-5,13-21.
Y	M. I. Garcia-Lloret <i>et al</i> "Demonstration of functional cytokine-placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion" <i>Experimental Cell Research</i> 214, 46-54 (1994). See pages 48-49, 51-53.	1-5, 13-21.

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